Effect of combined treatment with R-(+)-methanandamide and chemotherapeutic drugs in mantle cell lymphoma and chronic lymphocytic leukemia

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Abstract

Mantle cell lymphoma (MCL) is a non-Hodgkin B-cell lymphoma with very bad prognosis. The genetic hallmark of MCL, is the translocation t(11;14)(q13;q32) which leads to overexpression of cyclin D1, a D-type cyclin that is not usually expressed at high levels in normal B lymphocytes.

Previous studies indicate that cannabinoid receptors are expressed in lymphoma and have shown that lymphoma cell death is induced as a result of exposure to cannabinoids (ligands).

The aim of this diploma work was to combined cytostatics with the cannabinoid receptor ligand R (+)-Methanandmide (R-MA). Our data suggest that combination treatment with cytostatics and R-MA induces synergistic effects in most cases.
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonoyl glycerol</td>
</tr>
<tr>
<td>AEA</td>
<td>Arachidonoylethanolamide</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid receptor 1</td>
</tr>
<tr>
<td>CB2</td>
<td>Cannabinoid receptor 2</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>LR</td>
<td>Lower right-hand quadrant</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>R-MA</td>
<td>R(+)-Methanandmide</td>
</tr>
<tr>
<td>THC</td>
<td>Δ(9)tetrahydrocannabinol</td>
</tr>
<tr>
<td>Win55</td>
<td>(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)]</td>
</tr>
<tr>
<td>UR</td>
<td>Upper right-hand quadrant</td>
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1 Introduction

1.1 Mantle cell lymphoma (MCL)

Mantle cell lymphoma (MCL) is a lymphoid neoplasm which is characterized by an abnormal proliferation of mature B lymphocytes. MCL is characterized by short remissions and poor prognosis. The disease is rare and accounts for about 6% of all cases of non-Hodgkin lymphomas and mainly affects older men. MCL usually begins with lymph node enlargement but it can spread to other tissues such as the liver, bone marrow and gastrointestinal tract[1]. The median survival in MCL is about 3 years[2]. The first pathogenetic event in mantle cell lymphoma is a genetic change involving chromosome 11 and chromosome 14, called a “reciprocal translocation,” and abbreviated as t(11;14)(q13;q32)(Fig.1)[3]. This leads to an overexpression of cyclin D1, a D-type cyclin that is not usually expressed at high levels in normal B lymphocytes.

Figure 1. The genetic change involving chromosome 11 and chromosome 14 occurs in about 85% of the patients with MCL. That results in an overexpression of cyclin D1. Patients who do not overexpress cyclin D1 instead overexpress cyclin D2 or D3 [17].

1.2 Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is an abnormal neoplastic proliferation of B lymphocytes. CLL is the second most common type of leukemia in adults[4]. The symptom of CLL are enlarged lymph nodes like MCL, enlarged liver and spleen, fatigue, bone pain, excessive sweating, loss of appetite, weight loss e.g. The bone marrow is the starting point for CLL, but it can spread to blood, lymph nodes, spleen, central nervous system (CNS). The median survival of patients with CLL is around 10 years but the individual prognosis is highly variable. [5] D38 expression and the IgV_H gene mutational status are independent predictors of prognosis. Consequently, no association was found between CD38 expression and IgV_H gene mutation[18].
1.3 The Endocannabinoid system

Previous studies indicate that cannabinoid receptors are expressed in lymphoma and have shown that lymphoma cell death is induced as a result of exposure to cannabinoids (ligands).

The endocannabinoid system includes the cannabinoid receptors, endogenous cannabinoid ligands (endocannabinoids) and endocannabinoid-metabolizing enzymes. The name cannabinoid derives from the fact that cannabinoid receptors are the targets of D9-tetrahydrocannabinol (THC), the main psychotropic agent in the plant *Cannabis sativa* [19].

1.4 The Cannabinoid receptors and their ligands

Two different types of cannabinoid receptors have been identified and cloned in the early 1990s, the cannabinoid receptor 1 (CB1), and cannabinoid receptor 2 (CB2). CB1 is mostly located in the central nervous system but also in peripheral tissues, and CB2 mostly in the immune system. Low levels of CB2 have recently been detected in neuronal cells of the brain. Both CB1 and CB2 receptors transmit signals via inhibition of adenylyl cyclase and mitogen-activated protein kinases (MAPK) [7].

Two well studied endocannabinoids were identified some years after the cannabinoid receptors. The first endogenous cannabinoid ligand was isolated in 1992. It was arachidonylethanolamide (anandamide, AEA) (Fig 2) and the second was 2-arachydonoylglycerol (2-AG) (Fig 3) in 1995. The name arachidonylethanolamide derives from the Sanskrit word *ananda*, which means "bliss, delight". [8] Both are synthesized from phospholipids precursors. 2-arachydonoylglycerol (2-AG) has much higher serum and brain concentrations than anandamide [9].

![Fig 2. The structure of AEA][8].
Anandamide is a weak CB1 and CB2 agonist. Endocannabinoids are not stored in intracellular compartments like neurotransmitters, instead they are released upon demand from lipid precursors in the cytoplasmic membrane through enzyme activation[9].

Several synthetic cannabinoids have been engineered. Win-55 belongs to the substance group aminoalkylindoles, which is a non-selective agonist for both CB1 and CB2. There is another group called eicosanoids, which includes anandamide and analogues of anandamide such as R-(+)-methanandamide (R-MA)(Fig 4)[10]. R-MA is a metabolically stable analog of AEA [11].
1.5 Ceramide

Ceramide is a sphingolipid that regulates a variety of cellular processes including proliferation, differentiation and apoptosis and it comprises approximately 2% of the total sphingolipid content of the cell. In some cases, cannabinoids have been shown to induce accumulation of ceramide, which mediates growth inhibition and cell death (Fig 5)[10]. There are two major pathways from which ceramide can be produced, de novo ceramide synthesis and sphingomyelin degradation [10].

![Ceramide Diagram](image)

**Fig. 5.** First of all the cannabinoids bind to the CB1 and CB2 receptors. Signaling leading to apoptosis is mediated via the second messenger ceramide, which induces phosphorylation of p38. This in turn induces a loss of the mitochondrial membrane potential, which leads to caspase activation. Thereafter apoptosis takes place[10].

1.6 Chemotherapeutic drugs used in lymphoma treatment

Doxorubicin: Doxorubicin is a chemotherapy drug that is given as a treatment for many different types of cancer. Doxorubicin intercalates between base pairs in the DNA helix, thereby preventing DNA replication and ultimately inhibiting protein synthesis. Doxorubicin also inhibits replication by blocking an important enzyme called topoisomerase II[15].

Ara-C (Cytarabine): Ara-C belongs to the category of chemotherapy called antimetabolites. When the cells incorporate Ara-C into the cellular metabolism, they are unable to divide. Ara-C is cell-cycle specific. That means they attack cells in the S phase[20].
2 Materials and methods

2.1 Cell culturing

The mantle cell lymphoma cell lines RecC1, JekoC1 and the chronic lymphocytic Leukemia cell line MecC1 were grown at 37°C in 5 % CO\textsubscript{2} and humidified atmosphere. The RecC1 cell line was obtained from Dr. Christian Bastard, Ronan, France. MecC1 and JekoC1 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 μg of the antibiotic gentamicin. Both medium and supplements were from Invitrogen. All experiments were performed in serum-free AIM medium. This is due to the fact that R-MA is known to bind to serum, which would affect the results.

2.2 Cell viability assay

XTT (Biological Industries Kibbutz Beit) is a simple method for the determination of cell viability using standard micro plate absorbance readers. This method is mostly used to study growth factors and cytokines as well as screening for cytotoxic agents and lymphocyte activation. XTT is a tetrazolium derivative that measures cell viability based on the activity of mitochondria enzymes in live cells that reduce XTT and are inactivated shortly after cell death. [12] The viability tests were performed in AIM medium (Invitrogen). 200μl of cell suspension with different treatment was cultivated in a flat 96-well plate. The cells were incubated for 72 hours in 37°C and 5 % CO\textsubscript{2}. After the desired incubation period 50 μl XTT was added to each well and the plate was incubated for 4 hours. Afterward the absorbance was measured at a wavelength of 690 with an ELISA reader.

2.3 Cell death ELISA

Cell death ELISA (Roche, Mannheim, Germany) is a quantitative sandwich ELISA that detects histone and intranucleosomal DNA fragmentation by binding to 2 different monoclonal antibodies. The cells were washed and resuspended in AIM-V medium. 10 millions cells with different treatment that induce cell death were incubated for 24 h in 37°C and 5 % CO\textsubscript{2}. Lysis buffer was added and samples were incubated at room temperature for 30 min. The cells were transferred to 96-well plates. Anti-histone biotin and anti-DNA-POD and incubation buffer were added to each well and incubated for 2 hours in room temperature. Anti histone antibody reacts with the histones H1, H2A, H2B, H3 and H4 and anti-DNA POD antibody binds to single- and double-stranded DNA. After three washes with incubations buffer, the ABTS substrate was added for 20 minutes. Adding stop solution terminated the reaction, and the emission was determined at 405 nm[13][14].
2.4 Flow cytometric analysis

The distribution of the phospholipids of the plasma membrane is asymmetrical, this can be perceived between the inner and outer leaflets of the membrane. The substance phosphatidylserine is situated on the inner surface. A disruption of the asymmetry occurs during the apoptosis phase and phosphatidylserine becomes released on the outside surface of the plasma membrane. Due to the fact that the anticoagulant protein Annexin V binds with high affinity to phosphatidylserine, fluorochrome conjugated annexin has found a method as a marker of apoptotic cells. This is specifically utilised for their detection by flow cytometry. A reaction is caused within the cells with annexin V, and they lose the ability to exclude cationic dyes as PI in the plasma membrane. This draws the conclusion that by staining cells with a combination of annexin V and PI, there is likelihood to detect unaffected, non-apoptotic cells, early apoptotic cells and late apoptic cells.[21] Rec-1 and Jeko cells were treated with different treatment and harvested 24 h after treatment. Thereafter the cells were washed in PBS and resuspended in 100 µl binding buffer. 5µl of Annexin V-FITC and 5 ml of propidium iodide (PI) were added and incubated in dark for 15 min. Cells were then subjected to FACS analysis[16]. Annexin-V-PE aptosis Detection Kit (BD Biosciences Pharmingen) was used to detect apoptosis[10].
3 Results

Before using the XTT assay it is necessary to optimize the method for the specific cell line. This is done by measuring the absorbance for different dilutions of the cells. You choose the cell number to be used in further experiments by looking at the area in which the relationship between cell concentration and absorbance is linear. Rec-1, Jeko (MCL) and Mec-1 (CLL) were incubated for 24h in AIM-V medium and the cell proliferation was estimated using XTT assay. The optimal concentration of Mec-1, Rec-1 and Jeko cell lines was 0.5 million cells/ml (Fig 1). The range of cell concentrations was from $2 \times 10^6$ cells/ml to $7.8 \times 10^3$ cells/ml.

![Graphs of Mec-1, Rec-1, and Jeko cell lines showing viability at different concentrations after 24 h incubation in AIM-V medium measured with the XTT assay.](image_url)

Figure 1. Viability of the different cell lines at different concentrations after 24 h incubation in AIM-V medium was measured with the XTT assay.
In order to find the optimal dose of R-MA I performed a titration. Mec-1, Rec-1 and Jeko cells were treated with different dose of R-MA for 72 hours in AIMCV medium (Fig 2). The dose below 5 µM R-MA had no effect on viability. 10ul was chosen as the optimal dose, as it has earlier been shown that higher doses are not specific for the cannabinoid receptors.

![Graph showing viability of different cell lines after treatment with different doses of R-MA for 72 h in AIMCV medium measured with the XTT assay.](image)

Figure 2. Viability of the different cell lines after treatment with different doses of R-MA for 72 h in AIM-V medium measured with the XTT assay.

Titration was also performed in order to acquire the optimal dose of doxorubicin to be used in combination with R-MA. The different cell lines display a variable loss of viability after treatment with doxorubicin (Fig 3). The cell lines were treated for 72 h with increasing doses of doxorubicin. For further experiments I chose 200 and 300nm due to their high effects and the fact that higher doses of doxorubicin are known to be toxic to normal cells.

![Graph showing viability of different cell lines with different doses of treatment after 72 h incubation in AIM-V medium was measured with the XTT assay.](image)

Figure 3. Viability of the different cell lines with different dose of treatment after 72 h incubation in AIM-V medium was measured with the XTT assay.
The last titration was performed in order to acquire the optimal dose of Ara-C to be used in combination with R-MA. I chose 50nM and 100nM due to their high effects and the fact that higher doses of Ara-C are known to be toxic to normal cells. The different cell lines were seeded at a concentration of $5 \times 10^6$ cells/ml in a 96-well plate and exposed to different concentrations of cytarabin (AraC). The cell lines were treated for 72 hour in AIM-V medium with increasing doses of Ara-C (Fig 4).

![Figure 4. The different cell lines were treated with Ara-C and incubated for 72 hours in AIM-V medium. Cell proliferation was estimated by using XTT assay.](image-url)
In the first experiment the different cell lines were treated with 10 µM R-MA in combination with Ara-C (Fig 5). The concentration of Ara-C we used was 50 and 100 nM. Cells were incubated for 72h in AIM-V medium and the cell proliferation was estimated using XTT assay. R-MA and Ara-C induced synergistic in both Jeko and Rec-1 but not in Mec-1.

Figure 5. Cytotoxic effects of Ara-C were examined with and without combination of R-MA.
In the next experiment the cell lines were treated with 10 µM R-MA in combination with 200 nM or 300 nM of Doxorubicin (Fig 6). Cells were incubated for 72h in AIM-V medium and the viability was measured with the XTT assay. R-MA and doxorubicin showed synergistic effects in both Jeko and Rec-1 but not in Mec-1.

Figure 6. Cytotoxic effects of Doxorubicin were examined with and without combination of R-MA.
In order to investigate if the decreased viability was caused by cell death, the MCL cell lines Jeko and Rec-1 were treated with 10 µM R-MA in combination with Ara-C. Cells were incubated for 24h in AIM-V medium and were measured with the Flow Cytometric Analysis. The cells were labeled with Annexin V and propidium iodide. The concentration of Ara-C we used were 50 nM and 100 nM (Fig 7). R-MA and Ara-C induced additive effect in Rec-1 but not in Jeko.

Figure 7 Induction of cell death in MCL cell lines treated with Ara-C alone or in combination with R-MA. Flow cytometry analysis of Annexin V and propidium iodide was performed. Top and bottom right quadrants show late apoptotic/necrotic and early apoptotic cells, respectively.
Next the cell lines Jeko and Rec-1 (MCL) were treated with 10 µM R-MA in combination with 200 nM and 300 nM Doxorubicin. Cells were incubated for 24h in AIM-V medium. Thereafter the cells were labelled with Annexin V and PI and analyzed by flow cytometry (Fig 8). R-MA and Ara-C induced additive effect in Rec-1 but not in Jeko.

<table>
<thead>
<tr>
<th>Control</th>
<th>Jeko</th>
<th>R-MA</th>
<th>Rec-1</th>
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<tr>
<td></td>
<td></td>
<td>2.03%</td>
<td>19.55%</td>
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<td></td>
<td>99.98%</td>
<td>0.02%</td>
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<tr>
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<td>doxorubicin</td>
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<td>Annexin V FITC</td>
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<tr>
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<td>87.99%</td>
<td>5.11%</td>
<td>87.98%</td>
<td>5.11%</td>
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</tbody>
</table>

Figure 8 Jeko and Rec-1 (MCL) were treated with Doxorubicin alone or in combination with R-MA. Flow cytometry analysis of Annexin V and propidium iodide was performed. One representative cytogram showing early apoptotic cells that bind to Annexin V-FITC but exclude PI, lower right-hand quadrant (LR), and late apoptotic/necrotic cells that bind to both Annexin V-FITC and PI, upper right-hand quadrant (UR).
To investigate the amount of apoptosis after treatment with 10 µM R-MA in combination with Ara-C I performed cell death ELISA. MCL cell lines Jeko and Rec-1 were resuspended in AIM-V medium and treated with R-MA in combination with Ara-C for 24 h (Fig 8). Thereafter the cell death ELISA was performed, and fluorescence units are shown on the y-axis. We also treated Jeko and Rec-1 with R-MA in combination with Doxorubicin (Fig 9). R-MA in combination with both Ara-C and Doxorubicin induced additive effects on both Jeko and Rec-1 cell lines.

Figure 8. R-MA in combination with Ara-C induce cell death. The cell death ELISA was performed.

Figure 9. We combined the R-MA treatment with Doxorubicin, after 24 h the cell death was examined by cell death ELISA.
4 Discussion

Mantle cell lymphoma (MCL) is a moderately aggressive disease, which is not curable with chemo-immunotherapy. The median survival duration is short, approximately three years. Observations were made by Gustafson et al [10] that cannabinoids induce receptor-mediated apoptosis in mantle cell while normal cells remain unaffected. The use of cytostatics is an effective way in controlling tumour growth. It works by preventing the tumour cells from dividing and growing, and since tumour cells divide faster than normal cells cytostatics is very effective against these. Normally when treating patients with cytostatics you use a combination of several different cytostatics in order to attack cell division at different points of the process. The older patients cannot receive high doses of cytostatics without the potential of getting seriously ill. In order to deal with this problem I performed experiments in which I combined cytostatics with the synthetic cannabinoid R (+)-Methanandamide (R-MA). The two types of cytostatics we used in this studies (Ara-C and Doxorubicin) are often included in the treatment of lymphomas. The mantle cell lymphoma cell lines Rec-1, Jeko-1 and chronic lymphocytic leukemia cell line Mec-1 were treated with the R-MA with and without combination of chemotherapeutic drugs. My results suggest that a combination of a low dose of cytostatica with R-MA induces more cell death than just treatment with cytostatica. R-MA is known to induce cell death by apoptosis whereas cytostatics induces cell cycle arrest in the cells. And since the low dose of cytostatica wouldn’t affect older persons in a negative way, this combinatorial treatment could in theory be used in in vivo treatments in the future.

5 Acknowledgments

It is suitable to thank my supervisors for this study, Jenny Flygare and Kristine Gustafsson for giving me opportunity to perform my master project at Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet. I acknowledge Marcus Nordgren, my partner in the cell lab for sometimes splitting my cell lines and for all non scientific discussions down in the canteen. And last but not least I would like to give a special thanks to my examiner Professor Carl Påhlson.
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